Comparison of phenotypic methods for the detection of penicillinase in Staphylococcus aureus and proposal of a practical diagnostic approach

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Abstract: Objectives Disc diffusion is a cost-efficient, low-complexity, reliable method for detection of blaZ -mediated benzylpenicillin resistance in Staphylococcus aureus if the zone edge is inspected. EUCAST breakpoints cannot fully separate -lactamase-positive from -lactamase-negative strains, and EUCAST recommends the zone edge test. Literature on nitrocefin-based testing and the zone edge test is scarce with wide variations in reported assay performance. Methods This study compared two different nitrocefin-based commercial and in-house tests and the EUCAST-based zone edge test for penicillinase detection in S. aureus applying a PCR-based gold standard. Results In total, 215 non-duplicate clinical S. aureus isolates were included in the study, of which 127 (59.1%) did not harbour a blaZ gene, whereas 88 (40.9%) were blaZ positive. This study showed that for blaZ detection the zone edge test is more sensitive (96.6%) than nitrocefin tests independent of using nitrocefin discs (87.5% sensitivity) or solution (89.8% sensitivity), and that the significant inter-person variations of the zone edge test are probably related to the training level of the individual investigators (individual sensitivity ranging from 68.2% to 96.6%, specificity ranging from 89.8% to 100%). Conclusions In addition to continued and strict training of investigators, we propose mandatory checking of benzylpenicillin zone edges, particularly in an investigation zone from 26 to 30 mm, which can result in improved specificity/positive predictive value of the zone edge test (from 98.4% to 100%) but retains the high sensitivity/negative predictive value of the method.

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Comparison of phenotypic methods for the detection of penicillinase in *Staphylococcus aureus* and proposal of a practical diagnostic approach

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Objectives: Disc diffusion is a cost-efficient, low-complexity, reliable method for detection of *blaZ*-mediated benzylpenicillin resistance in *Staphylococcus aureus* if the zone edge is inspected. EUCAST breakpoints cannot fully separate β-lactamase-positive from β-lactamase-negative strains, and EUCAST recommends the zone edge test. Literature on nitrocefin-based testing and the zone edge test is scarce with wide variations in reported assay performance.

Methods: This study compared two different nitrocefin-based commercial and in-house tests and the EUCAST-based zone edge test for penicillinase detection in *S. aureus* applying a PCR-based gold standard.

Results: In total, 215 non-duplicate clinical *S. aureus* isolates were included in the study, of which 127 (59.1%) did not harbour a *blaZ* gene, whereas 88 (40.9%) were *blaZ* positive. This study showed that for *blaZ* detection the zone edge test is more sensitive (96.6%) than nitrocefin tests independent of using nitrocefin discs (87.5% sensitivity) or solution (89.8% sensitivity), and that the significant inter-person variations of the zone edge test are probably related to the training level of the individual investigators (individual sensitivity ranging from 68.2% to 96.6%, specificity ranging from 89.8% to 100%).

Conclusions: In addition to continued and strict training of investigators, we propose mandatory checking of benzylpenicillin zone edges, particularly in an investigation zone from 26 to 30 mm, which can result in improved specificity/positive predictive value of the zone edge test (from 98.4% to 100%) but retains the high sensitivity/negative predictive value of the method.

Introduction

Penicillin-resistant *Staphylococcus aureus* were described soon after benzylpenicillin had entered clinical practice.[1–3] In Switzerland, the prevalence of benzylpenicillin-resistant *S. aureus* (non-MRSA) has been reported to have remained stable at about 80% for the last 5 years.[4] Despite this high number of resistant isolates, benzylpenicillin nevertheless remains of clinical importance due to its few side effects, strong bactericidal effect, wide dosing range, targeted spectrum of activity and low therapeutic costs. In the University Hospital of Zurich that is served by the Institute of Medical Microbiology, University of Zurich, benzylpenicillin is, therefore, regularly used, if the organism tests as susceptible, e.g. for *S. aureus*.

Disc diffusion is considered more reliable than MIC testing for the detection of *blaZ*-mediated benzylpenicillin resistance.[5] However, EUCAST also states that the benzylpenicillin clinical zone diameter breakpoint of 26 mm (which equals the epidemiological cut-off, i.e. the ECOFF) will not completely separate β-lactamase producers (non-WT) from β-lactamase non-producers (WT), i.e. both populations overlap and the ECOFF is not discriminatory.[5,6] EUCAST has, therefore, been recommending the zone edge test in addition to clinical breakpoints (CBPs) from its first guideline versions on, and, in addition, explicitly discourages the use of chromogenic cephalosporin-based β-lactamase tests in its most recent guidelines (version 6.0, similar to current CLSI 2016 guidelines) starting with EUCAST breakpoint tables version 2.0.[5,7–9] The available literature on nitrocefin-based penicillinase testing and the zone edge test is, however, scarce with wide variations in the reported assay performance parameters. Although more recent studies triggered the latest EUCAST recommendations, the original zone edge criterion was proposed as long ago as 1981.[10] CLSI

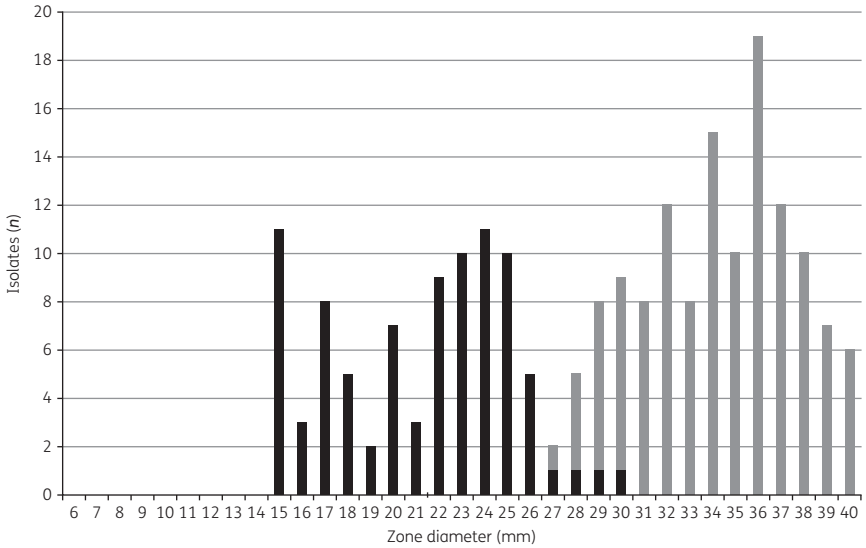

Figure 1. Distribution of benzylpenicillin 1 U disc zone diameters for 215 *S. aureus* strains studied and genotype as determined by *blaZ* PCR (black bars, *blaZ*-positive strains; grey bars, *blaZ*-negative strains).

guidelines contain recommendations for inspection of the penicillin zone edge that are similar to EUCAST even though disc potency and zone diameter CBP differ.[7]

The aims of our study were: (i) to compare different chromogenic nitrocefin-based commercially available and in-house tests for the detection of penicillinase activity in *S. aureus*; (ii) to determine the extent of investigator-dependence and the influence of methodological variables on the zone edge test to elucidate the wide variations in reported sensitivity; and (iii) to develop a practical approach to optimize the application of the zone edge test in a routine clinical laboratory.

Materials and methods

Bacterial isolates

In total, 215 non-duplicate clinical *S. aureus* isolates recovered in our clinical laboratory from 2010 until 2015 were included in the study (Table 1). For all isolates MRSA was excluded by cefoxitin susceptibility according to the current EUCAST/CLSI breakpoint (≥22 mm).[5,7] Isolates displaying benzylpenicillin zone diameters ranging from 15 to 40 mm were selected as a representative sample (Figure 1). Isolates with benzylpenicillin zone diameters <15 mm were not included as production of a penicillinase can be assumed with high probability. All isolates were genetically characterized for the presence of *blaZ* penicillinases (types A, B, C and D, representing the worldwide genetic variation). 127/215 isolates (59.1%) did not harbour a *blaZ* gene, whereas 88/215 isolates (40.9%) were *blaZ* positive. All isolates were subcultured twice on Columbia sheep blood agar (bioMérieux, Marcy-l'Étoile, France) prior to performing the assays.

Genetic detection of blaZ genes

Total DNA was extracted from bacterial colonies after growth on sheep blood agar using the InstaGene Matrix (Bio-Rad, Reinach, Switzerland). Genetic detection of *blaZ* genes was done by two classical endpoint PCRs for all 215 isolates and primers to cover all *blaZ* subtypes (A, B, C and D, representing the worldwide genetic variation): one PCR followed an in-house protocol and covered subtypes A, C and D; and a second PCR detecting subtype B was done as described elsewhere.[11] The in-house *blaZ* PCR was performed using 5 μL of 10× FastStart PCR buffer with 20 mM $MgCl_2$ (Roche Diagnostics, Rotkreuz, Switzerland), 5 μL of dNTP-Mix (2 mM, in-house, 3× dUTP), 1 μL of primers *blaZ*-fwd (5′-CAACGTCTAAAAGAACTAGG-3′) and *blaZ*-rev (5′-CCTTCATTACACTCTTGG-3′) amplifying a 418 bp fragment (nucleotide position 420–838), 0.25 μL of FastStart Taq DNA polymerase (5 U/μL, Roche), 5 μL of DNA extract and 32.75 μL of molecular grade water (Roche) in a total reaction volume of 50 μL. The PCR protocol comprised an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 48 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were separated in a 1.1% agarose gel (Invitrogen AG, Basel, Switzerland) in 0.5× Tris-borate-EDTA buffer at 120 V for 50 min and stained with GelRed (Chemie Brunschwig, Basel, Switzerland). A 100–1500 bp ladder (XIV, Roche) was used to determine the size of amplicons. *gyrB* amplification was used as an amplification control as described by other authors.[12] In each PCR run control strains were included: *S. aureus* ATCC 29213 served as a positive control for *blaZ*, while *S. aureus* ATCC 25923 was used as a *blaZ*-negative control strain.

Susceptibility testing

Disc diffusion susceptibility testing was done according to EUCAST recommendations.[5] Antibiotic discs were obtained from i2a, Montpellier, France, and Mueller–Hinton II agar was obtained from Becton Dickinson, Franklin Lakes, NJ, USA. Inhibition zone edges of benzylpenicillin 1 U discs were read by nine investigators with different levels of training and experience in the inspection of benzylpenicillin zone edges, ranging from a comprehensive basic introduction that is given to all new readers in our laboratory using the EUCAST reading guide including relevant images (investigator 8 representing an entry level), or long-standing experience but no current daily routine use (investigators 1, 2 and 4) up to highly experienced and daily routine-trained investigators (investigators 3, 5–7 and 9).[13] Investigators were either technicians with different levels of professional experience (investigators 5, 6 and 7 had laboratory practice of ≥20 years and investigators 1 and 9 had laboratory practice of 5–10 years), or represented academic medical microbiologists (investigators 3 and 4 had laboratory practice of ≥20 years, investigator 2 had laboratory practice of 12 years and investigator 8 had diagnostic laboratory practice of 4 years). Investigators were asked to read in their normal way and zone reading followed EUCAST recommendations, which are based on the initial descriptions of Gill *et al.*[10] In brief, benzylpenicillin zone edges were

Table 1. Performance of nitrocefin discs, nitrocefin solution and benzylpenicillin zone edge test for the detection of penicillinase production in *S. aureus*[a]

Method	Magnifying lens used (Y/N)	Level of overall experience[b]	Level of daily practice[c]	TP	FP	TN	FN	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CBP only				79	0	127	9	215	89.8	100	100	93.4
Nitrocefin disc[d]				77	0	127	11	215	87.5	100	100	92.0
Nitrocefin solution[d]				79	0	127	9	215	89.8	100	100	93.4
Zone edge/ investigators												
1	Y	++	++	85	2	125	3	215	96.6	98.4	97.7	97.7
2	N	++	+	85	13	114	3	215	96.6	89.8	86.7	97.4
3	Y	+++	++	85	0	127	3	215	96.6	100	100	97.7
4	N	+++	+	84	13	114	4	215	95.5	89.8	86.6	96.6
5	Y	+++	++	85	0	127	3	215	96.6	100	100	97.7
6	Y	+++	++	85	0	127	3	215	96.6	100	100	97.7
7	Y	+++	+++	85	1	126	3	215	96.6	99.2	98.8	97.7
8	N	+	+	60	2	125	28	215	68.2	98.4	96.8	81.7
9	Y	++	++	84	1	126	4	215	95.5	99.2	98.8	96.9

[a]PCR results were used as the gold standard; TP, true-positive; FP, false-positive; TN, true-negative; FN, false-negative.
[b]Level of overall experience: +, <5 years; ++, 5–15 years; +++, >15 years.
[c]Level of daily practice: +, occasional zone reading; ++, regular but not daily zone reading; +++, daily zone reading.
[d]Inconclusive results were rated positive.

inspected using transmitted light in front of a dark background, i.e. angular transmitted illumination by a lamp in front of a black laminated board; investigators 2, 4 and 8 used the naked eye in a distance of about 30 cm as recommended by EUCAST, whereas investigators 1, 3, 5–7 and 9 used a magnifying glass (currently discouraged by EUCAST).[13] Zone edges were classified as sharp if they were well defined and—in some instances—fully developed colonies were visible within the inhibition zone close to the zone edge. Zone edges were defined as fuzzy if they were not clearly delimited, showing gradual tapering of growth.

Nitrocefin disc test

Nitrocefin discs (BBL™ Cefinase™ Paper Disks) were purchased from Becton Dickinson, Franklin Lakes, NJ, USA and used according to the recommendations of the manufacturer [package insert 8800801JAA(02), 2015-04]. Briefly, discs were moistened with one drop of purified water and colony material picked from the cefoxitin inhibition zone edge on Mueller–Hinton II agar to foster *blaZ* expression was smeared onto the discs using a sterilized loop. Discs were checked for colour change after 60 min. Colour change from light yellow to orange/red was rated as a positive result; no colour change was rated as a negative result. If it was unclear whether a colour change had happened, the test was repeated three times, and if still unclear the result was recorded as inconclusive and considered positive for the calculation as in clinical practice it seems reasonable to report doubtful penicillin susceptibility tests as resistant. *S. aureus* ATCC 29213 served as a positive control, while *S. aureus* ATCC 25923 was used as a negative control strain for each run.

Nitrocefin solution test

Nitrocefin powder was purchased from Becton Dickinson, Franklin Lakes, NJ, USA (for research use only), dissolved in purified water to a concentration of 0.5 mg/mL, and aliquotted in sterile plastic tubes (100 μL, i.e. 50 μg/tube) and stored at −20 °C. Aliquots were thawed and adjusted to room temperature immediately before starting the tests. One loop of colony material picked from the cefoxitin inhibition zone edge on Mueller–Hinton II agar to foster *blaZ* expression was resuspended per nitrocefin tube, and tubes were incubated at 37 °C for 60 min and subsequently checked for colour changes. Colour changes from light yellow to orange/red were rated as a positive result; no colour change was rated as a negative result. If it was unclear whether a colour change had happened, the test was repeated three times, and if still unclear the result was recorded as inconclusive and considered positive for the calculation. *S. aureus* ATCC 29213 served as a positive control, while *S. aureus* ATCC 25923 was used as a negative control strain for each run.

Software

All calculations were done using Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA).

Results

Benzylpenicillin zone diameters and detection of blaZ

Of the 136 *S. aureus* isolates displaying benzylpenicillin zone diameters ≥26 mm (i.e. EUCAST ECOFF and CBP), 127 (93.4%) were *blaZ* negative, whereas *blaZ* was detected in 9 isolates (6.6%; Figure 1). All 79 isolates with benzylpenicillin zone diameters <26 mm were found to be *blaZ* positive. The sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) of the EUCAST ECOFF/CBP for the detection of the *blaZ*-positive non-WT were thus 89.8%, 100%, 100% and 93.4%, respectively (Table 1). Two isolates with a benzylpenicillin zone diameter ≥26 mm were found to be *blaZ* PCR positive (repeated three times from independent colonies and independent DNA extractions), but negative in the nitrocefin assays and considered to display a fuzzy zone edge by all nine investigators. To resolve these discrepancies, we conducted a cloverleaf test using *S. aureus* ATCC 25923 as the indicator strain as reported by other authors.[14] Both strains yielded a clearly positive result in the

cloverleaf test and were therefore considered true-positive for the presence of *bla*Z.

Nitrocefin disc test

The sensitivity of the nitrocefin disc was 87.5% if the four inconclusive/borderline results were considered positive, whereas specificity was 100% (Table 1). The resulting NPV for the penicillinase detection was 92%.

Nitrocefin solution test

The sensitivity of the nitrocefin solution test was 89.8% if the four inconclusive/borderline results were considered positive, whereas specificity was 100% (Table 1). The resulting NPV for the penicillinase detection was 93.4%.

Zone edge test

Although the median sensitivity and specificity, PPV and NPV of the zone edge test for penicillinase detection were high, ranging up to 96.6%, 100%, 100% and 97.7%, significant inter-investigator differences were observed (Table 1): the sensitivity of the zone edge test ranged from 68.2% for investigator 8 to 96.6% for investigators 1–3 and 5–7. Specificity varied between 100% for investigators 3, 5 and 6 and 89.8% for investigators 2 and 4. The 1-fold standard deviation of sensitivity and specificity for all nine investigators was, thus, 9.4% and 4.3%, respectively. While investigators with a comparably high level of overall experience yielded high sensitivities of 95.5%–96.6%, the less-experienced investigator 8 had a sensitivity of 68.2%. However, even for experienced investigators specificities varied with their level of current daily practice, ranging from 89.8% (investigator 4: overall laboratory experience of >20 years, occasional zone reading, no magnifying glasses used), to 98.4% (investigator 1: overall laboratory experience of 10 years, regular but not daily zone reading, magnifying glasses used), to 99.2% (investigator 7: overall laboratory experience of 20 years, daily zone reading, magnifying glasses used), up to 100% (investigators 3, 5 and 6: overall laboratory experience each of ≥20 years, regular but not daily zone reading, magnifying glasses used; Table 1). Thus, the best sensitivities and specificities were yielded by experienced investigators in daily practice and using magnifying glasses.

Discussion

The benzylpenicillin ECOFF and the equal EUCAST CBP of 26 mm do not reliably exclude the presence of *blaZ* (see Figure 1 and Table 1). The NPV of the EUCAST CBP of benzylpenicillin, i.e. the correct assignment of an isolate to the *blaZ*-negative, benzylpenicillin-susceptible WT, was 93.4% in this study, leaving a significant risk of therapeutic failure. Thus, EUCAST recommends using the zone edge test as an additional criterion.[5,7] The use of nitrocefin-based assays for the confirmation of penicillin susceptibility is explicitly discouraged. EUCAST recommendations are based on a limited number of studies investigating the performance of nitrocefin-based assays for *blaZ* detection, which show low sensitivity of these tests ranging from 35.7% to 63%.[14–16] Our results confirm other reports of nitrocefin assays displaying lower sensitivity than the zone edge test, although the sensitivity of both the disc- and the solution-based nitrocefin test were higher in this study as compared with others (87.5%–89.8%; Table 1). These differences in sensitivity may be explained by different study populations or by the picking of colony material from the zone edge of the cefoxitin inhibition zone in our study, fostering *blaZ* expression and subsequent enhancement of nitrocefin cleavage.[17] The use of nitrocefin solution instead of discs did not significantly increase sensitivity (increase of 2.3%, Table 1). The additional working time for preparation of the nitrocefin solution tubes and the higher hands-on time in performing the assay can probably not be justified by the limited performance increase.

The zone edge test is proposed to display the best performance; however, reported sensitivities are highly variable, ranging from 65.5% to 100%.[14–16] This wide variation in reported performance may be due to the assessment of the zone edge depending significantly on the investigator carrying out the inspection (see Table 1). Our results clearly show that the zone edge test has the potential to be performed with very high sensitivity and specificity: to achieve acceptable sensitivity it is, however, essential to continuously train readers using this test in addition to providing a comprehensive introduction. In addition, the EUCAST recommendation of not using magnifying glasses may need to be revised as the trained investigators in this study used magnifying glasses (Table 1) and achieved excellent results.[13] Furthermore, it seems of critical importance to follow EUCAST recommendations to ensure adequate reading, i.e. examining the zone edges using transmitted light as stated in the EUCAST disc diffusion manual.[18] The EUCAST manual states that plates should be 'held up to light'. In addition, the use of a generally dark background in contrast to angular transmitted light was considered helpful by the investigators in this study.

To increase specificity and to better standardize the zone edge test, it would be of advantage even for experienced investigators to limit zone edge testing to that particular diameter range of benzylpenicillin in which the *blaZ*-positive population and the *S. aureus* WT population overlap (investigation zone). In our study this was the case for a diameter range of 26–30 mm (Figure 1). The uppermost zone diameter value of the non-WT (30 mm), which has been proposed as resistant population cut-off (RCOFF), serves as the cut-off for additional testing, i.e. zone edge.[19] If this rule is followed, a simple and practical flow chart results (Figure 2), which classifies confirmed *blaZ* producers, WT and a borderline population according to the penicillin zone diameter. If this algorithm had been applied, the specificity of individual investigators would have been increased from 89.8% to 98.4% and 99.2% (investigators 2 and 4, respectively, who had less daily practice), or from 98.4%, 98.4% and 99.2% to 100% (investigators 1, 8 and 9, respectively, with more daily routine practice). Importantly, the sensitivity remained generally unchanged with the application of the investigation zone.

Limitations of this study are the restriction to one centre, one regional population of *S. aureus* strains investigated, and the use of media and discs from single manufacturers. A multi-laboratory, geographically diverse study is needed to confirm these regional findings. Furthermore, our results are primarily applicable only to the EUCAST disc diffusion method as CLSI uses a different disc load (10 U) versus 1 U (EUCAST) and, consequently, uses a different breakpoint. Furthermore, the number of isolates with a benzylpenicillin zone diameter ≥26 mm and a fuzzy zone edge (nine isolates, i.e. 6.6%) was comparably low.

In summary, there are five conclusions that can be drawn from this study: (i) our data confirm the EUCAST ECOFF and CBP as well

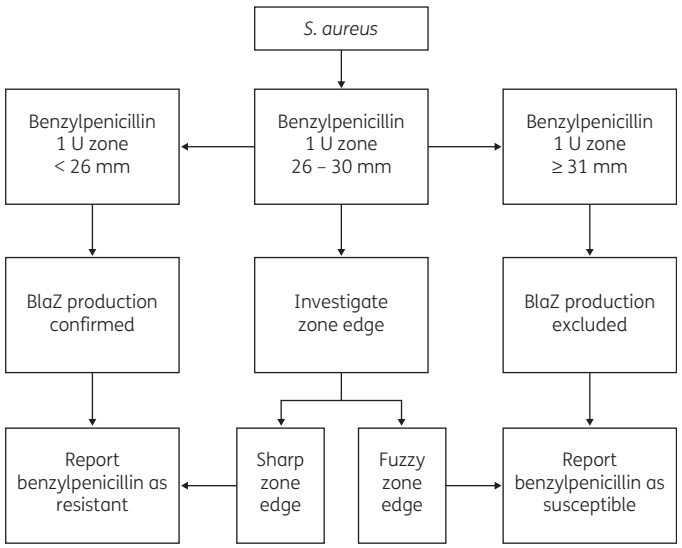

Figure 2. Proposed diagnostic flow chart for benzylpenicillin 1 U EUCAST testing and reporting in *S. aureus*.

as the lack of sensitivity and specificity of these cut-offs, which necessitate an additional test for penicillinase detection; (ii) the zone edge test is more sensitive than nitrocefin tests irrespective of whether nitrocefin disc or solution is used—therefore in line with EUCAST recommendations the nitrocefin tests should not be used; (iii) the zone edge test shows significant investigator dependence as demonstrated by varying sensitivity and specificity; (iv) thorough training and continued practice is important to achieve an acceptable performance in the zone edge test and magnifying glasses may improve investigator performance; and (v) an investigation zone from 26 to 30 mm and the EUCAST benzylpenicillin 1 U disc could improve the specificity/PPV of the zone edge test whilst retaining its high sensitivity/NPV.

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None to declare.

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